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Conan K. Wang, David J. Craik

Institutions: University of Queensland

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Designing macrocyclic disulfide-rich peptides for biotechnological applications

Conan K. Wang, David J. Craik

Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland, 4072, Australia

*Corresponding Author:

Professor David J. Craik

Institute for Molecular Bioscience,

The University of Queensland,

Brisbane, Qld, 4072, Australia

Tel: 61-7-3346 2019

Fax: 61-7-3346 2101

e-mail: d.craik@imb.uq.edu.au

Abstract

Bioactive peptides have potential as drug leads, but turning them into drugs is a challenge because of their typically poor metabolic stability. Molecular grafting is an approach for stabilising and constraining peptides and involves melding a bioactive peptide sequence onto a suitable molecular scaffold. It has the benefit of improving the stability of the bioactive peptide lead and potentially expanding its functionality. Here we step through the molecular grafting process and describe successes and limitations. So far, molecular grafting has been successfully used to improve the stability of peptide drug leads, enhance conformational rigidity, facilitate delivery to intracellular targets, and in some cases, increase efficacy in oral administration. Although applications of molecular grafting have focused mainly on therapeutic applications, including for pain, metabolic disease, and cancer, its potential uses are much broader and we hope this Perspective will inspire wider applications of this molecular design tool in biotechnology.

~~1. Peptides as drugs or molecular tools~~

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Peptides, which we consider here to comprise fewer than 50 amino acids, have a wide range of applications in chemical biology because they offer the selective and potent bioactivity of proteins but have some advantages because of their smaller size[1,2]. ~~(We arbitrarily consider peptides to comprise fewer than 50 amino acids).~~ However, despite their potential, peptides have certain limitations to their applicability, including poor stability. So, how do we overcome these deficiencies to capture the value of bioactive peptides ~~by overcoming their deficiencies~~? One answer is to graft them onto molecular scaffolds that have privileged biopharmaceutical properties[3-10]. In this Perspective, we focus on peptide scaffolds that are rich in disulfide bonds and have rigid, well-defined structures. More importantly, these peptide scaffolds are backbone-cyclized and hence have no free ends for proteases to attack.

Cyclic disulfide-rich peptides

The 'scaffold' peptides we focus on here are cyclic disulfide-rich peptides from plants and animals, including those that occur naturally and those that have been synthetically engineered (**Fig. 1a**). In order of increasing size, the natural cyclic peptides include sunflower-derived peptides of 12–14 amino acids with a single disulfide bond[11], θ-defensins of 18 amino acids with three disulfide bonds in a laddered arrangement[12] and cyclotides of around 30 amino acids with three disulfide bonds in a knotted configuration[13]. All of these natural peptides are ribosomally synthesized as linear precursors and then post-translationally processed into their mature cyclic forms in their host organisms[14]. There, they typically have roles as host defence agents, and we assume that their ultrastable cyclic structures have evolved because of the advantages they provide of stability, potency and adaptability to

changing pest or pathogen pressures. Some of these advantages can be transferred to acyclic peptides to engineer artificial cyclic scaffolds by adding amino acid linkers to bridge their termini; some examples of these engineered peptides include cyclic conotoxins of 22 amino acids with two disulfide bonds[15] and the cyclic chlorotoxin of 43 amino acids with four disulfide bonds[16].

Cyclic disulfide-rich peptides have both a macrocyclic backbone and one or more disulfide bonds. The disulfide bonds are typically conserved within each class of these peptides, and so provide scaffolds onto which the peptide sequences between successive Cys residues, called 'loops', are elaborated. Many of these loops have high sequence variability; a sequence alignment of cyclotides indicates that almost all non-Cys positions are tolerant to substitution (**Fig. 1b**). Although this type of analysis ignores correlated interactions between residues, the large (and expanding) diversity of sequences within each peptide class supports the idea that cyclic disulfide-rich peptides can tolerate substantial change to their sequences – a prerequisite if we wish to engineer them.

One of the main attractive biopharmaceutical properties of cyclic disulfide-rich peptides is their stability. For example, kalata B1 is stable in conditions that would normally degrade or denature ~~‘regular’~~linear peptides within minutes, such as the presence of chaotropic agents or acid and at temperatures approaching boiling, ~~and in acid,~~ as well as following incubation with proteases, such as trypsin, endoproteinase Glu-C, or thermolysin, and in human serum[17]. High serum stability is characteristic of other disulfide-rich cyclic peptides as well, including SFTI-1 (ref. 18), RTD-1 (ref. 19), cVc1.1 (ref. 15) and cyclic chlorotoxin[16]. The combination of a macrocyclic backbone and disulfide bonds underpins this proteolytic stability[15,20-22].

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A second advantage of cyclic disulfide-rich peptides is that some can penetrate cells and thus be used as intracellular delivery scaffolds[23-26]. Kalata B1, which internalizes into mammalian cells in amounts comparable to the cell-penetrating peptide TAT[26], enters cells via both endocytosis and direct membrane translocation, with entry initiated by binding to surface-exposed phosphatidylethanolamine lipids. Entry of MCoTI-II, meanwhile, is mediated by an endocytic pathway involving interaction of its positively charged residues with phosphoiniositides at the membrane surface[23,25,27].

Finally, many cyclic disulfide-rich peptides have intrinsic bioactivities that make them good leads for drug development or for agricultural applications. For example, θ-defensins have antimicrobial activities, the cyclic conotoxin cVc1.1 has potent analgesic activity, and cyclic chlorotoxin can preferentially bind to tumour cells. Additionally, natural cyclotides have insecticidal, antiviral, antimicrobial, nematocidal and molluscicidal activities, all of which could be relevant to the protection of crop plants from microbes, pathogens or herbivorous pests[13]. Nevertheless, our focus here is not so much on the intrinsic activities of these molecules but on how one might harness the desirable biopharmaceutical properties of cyclic disulfide-rich peptides to develop new drugs designed to have a specific activity, as described below.

Principles of ~~M~~molecular grafting

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The concept of molecular grafting is akin to its namesake in horticulture, i.e., the practice of fusing the scion of one plant to the stock of another, thus creating a new organism that has

desired properties of both starting plants. In molecular grafting the aim is to make a new peptide by grafting (*i.e.,* inserting) a linear peptide sequence of natural or synthetic origin, which has a desired biological activity, into a disulfide-rich peptide, which has desired biopharmaceutical properties, such as stability or oral bioavailability (**Fig. 2**). Note that we refer to the linear peptide sequence as the 'epitope', adopting a broader meaning than its definition as an antigenic determinant in immunology. We refer to the disulfide-rich peptide that accommodates the epitope as the 'scaffold' because it is the moiety able to display various analogues of the epitope. The entire epitope–scaffold fusion is called the 'grafted peptide'.

Tables 1 and **2** summarize recent grafting studies that have used cyclic disulfide-rich peptides as scaffolds, demonstrating their successful applications in a wide range of diseases, including cardiovascular disease, metabolic disease, immunological disease, pain and cancer. The data in the tables demonstrate that many different epitopes are amenable to molecular grafting and that cyclic disulfide-rich peptides can tolerate many different and substantial changes to their sequences. Two main strategies have been employed to design grafted peptides; i.e., those involving chemical processes, such as residue mutagenesis (**Table 1**); or those involving library-based processes (**Table 2**), such as recombinant display and screening of grafted peptide libraries. For either of these approaches there are three main ~~reasons~~ motivations for exploring a molecular grafting strategy: stabilization of peptide epitopes, improving activity, and improving intracellular delivery.

An approach for stabilizing peptide epitopes. An unequivocal finding from grafting studies is that stability of linear peptide epitopes in human serum can be enhanced by grafting them

onto a stable scaffold (**Fig. 3a**)[28-32]. For example, an epitope from myelin oligodendrocyte glycoprotein (MOG) that has potential use in the treatment of multiple sclerosis is rapidly degraded in serum (<1 hr), but becomes resistant to degradation once grafted onto kalata B1 (surviving for >24 hr)[28]. Similarly, grafting pro-angiogenic epitopes onto SFTI-I or MCoTI-II[30], and bradykinin B_1 receptor antagonists onto SFTI-I[32] improved their stability in human serum. Grafted cyclic peptides are typically poor substrates for a wide range of proteases, including pancreatin[28], thrombin[30] or trypsin[32].

Grafted peptides can also inherit other stability characteristics of the parent scaffold, including resistance to heat or acidic conditions. A grafted peptide comprising a MOG-derived epitope remained largely intact in strong acid after 24 hrs, whereas the epitope itself was completely hydrolysed after 6 hrs[28]. Similarly, a grafted SFTI-I containing an immunomodulatory sequence exhibited high thermal stability and maintained structural integrity up to 85 °C[33]. Grafted peptides are typically highly stable because they retain the stabilizing structural features of the scaffold – a cyclic backbone and a reinforcing network of disulfide bonds.

An approach for improving activity. Grafted peptides have demonstrated improved affinity and selectivity for target receptors. For example, improved binding affinity to $\alpha_9\beta_1$ integrin was reported for a heptapeptide from osteopontin upon grafting onto either SFTI-1 or MCoTI-II[30]. Furthermore, increased receptor sub-type selectivity of a melanocortin receptor-4 agonist was achieved by grafting it onto kalata B1 (ref. 34). The improvement in activity from conformational constraint probably has a thermodynamic origin, such as by minimising entropic losses on binding[35], but the effects of constraints on binding enthalpy and entropy

are complex and difficult to predict[36]. The advantage of having a suite of scaffolds for grafting, with selected examples shown in **Figure 1**, is the availability of a range of pre-formed structural elements that can readily accommodate the epitope and test the effect of constraining it. In the MOG study, the epitope was grafted into the β-turn of kalata B1 to mimic its native conformation[28]. In cases where the selected scaffold does not have a known pre-existing structure, additional residues flanking the epitope can be used as structural facilitators. For example, in a recent study the bioactive α-helical conformation of a grafted epitope was favoured by conjugation to a helix-stabilizing adapter sequence derived from apamin, a bee-venom peptide[37].

An approach for ~~intracellular~~ targeting and delivery ~~and improving oral activity~~. To deliver a bioactive peptide across the cell membrane and into the intracellular space, scaffolds that exhibit cell-penetrating properties can be used (**Fig. 3b**). A grafted peptide based on the cell-penetrating cyclotide MCoTI-I successfully modulated intracellular levels of p53 and associated transcriptional targets specifically in p53-expressing cells, confirming that traversal of the cell membrane had occurred[37]. In another study, an epitope that antagonizes the protein SET was grafted onto MCoTI-II, a cell-penetrating cyclotide similar in sequence to MCoTI-I[38]. Intracellular activity was monitored using GFP fluorescence as a readout for NF-κB-dependent gene expression, and the grafted peptide led to concentration-dependent transcriptional responses, demonstrating effective intracellular delivery. Recent efforts to enhance cellular uptake of scaffolds through grafting and chemical design promise to further expand the potential of cyclic disulfide-rich peptides as delivery scaffolds, whose inherent stability already sets them apart from traditional linear cell-penetrating peptides[39].

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Cyclic disulfide-rich peptides have in some cases shown orally deliverable activity (**Fig. 3c**)[15,32,40,41]. For example, the linear conotoxin Vc1.1 is analgesic against neuropathic pain in rats when injected, but not when administered orally; however, a backbone-cyclized version of this conotoxin is orally active[15]. Another example is the grafting of a peptidic bradykinin B_1 receptor antagonist sequence onto SFTI-1 or kalata B1 scaffolds, which resulted in orally active peptides for the treatment of inflammatory pain, while the linear antagonist sequence alone exhibited essentially no oral activity[32,40]. Similarly, a point-mutated kalata B1 scaffold was reported to be orally active in a dose-dependent manner in animal models of multiple sclerosis and resulted in lowered inflammation and reduced areas of axonal demyelination compared to untreated controls [41]. We caution that we do not believe that cyclisation alone is sufficient to induce oral activity in a peptide. The factors that contribute to oral uptake are multifarious and not fully understood, and this is an area of much current investigation[42-45]. Nevertheless, there are now several man-made examples of peptides with high oral activity and/or bioavailability[42,46,47].

The process of molecular grafting

We now describe the molecular grafting process, highlighting key points to be considered, based on lessons learnt in our laboratory and those of others. First, molecular grafting requires the selection of a suitable epitope and scaffold pair, followed by structural and functional characterization, as illustrated in **Figure 4**. Experimentally, molecular grafting involves a range of techniques drawn from chemistry, biophysics, and biology.

The nature of the epitope and its mechanism of action can affect the likelihood of success. Most importantly, the termini of the epitope should not be essential for its activity, as

eventually those termini will be integrated into the scaffold. For example, many peptide substrates of PDZ domains, a common structural motif of signalling proteins, require their C-termini to bind to their cognate targets and so would make poor starting epitopes for grafting. Indeed, a peptide substrate of the PDZ2 domain of the postsynaptic density-95 protein could no longer bind its target after it was grafted onto SFTI-1, but binding could be rescued by incorporation of an Asp residue to mimic a free C-terminus[48]. That grafting study, along with others[49,50], are examples of using molecular grafting to target protein pockets. Targeting of flat surfaces is also possible, as exemplified by the successful design of grafted peptides that bind the CD58 adhesion protein[33].

The size of the epitope is an important consideration, with short epitopes (<10 amino acids) typically easier to handle. Epitopes ranging from a single amino acid[41] (smallest graft possible) to 21 amino acids[38] have been grafted onto cyclotides, whereas grafts up to 12 (ref. 33), 15 (ref. 48) or 16 (ref. 51) amino acids have been successfully incorporated into the RTD-1, SFTI-I, and cVc1.1 scaffolds, respectively. In cases where the size of an epitope is prohibitively large, a smaller region of the epitope can be used instead, provided that it substantially contributes to the binding energy of the interaction[52]. Identification of such a truncated epitope can be achieved from mutation–activity studies and/or structures of the epitope bound to its target[33].

With these considerations in mind, peptide epitopes can be selected from three major sources: (*i*) naturally occurring peptides or fragments of an interacting protein, (*ii*) chemical peptide libraries or (*iii*) recombinant peptide libraries. As an example involving a naturally occurring peptide, the design of an orally active peptide for inflammatory pain was achieved by grafting

an epitope derived from kallidin, an endogenous allogenic peptide, onto kalata B1 (ref. 40). To design a potent inhibitor of kallikrein-related peptidase-4, a potential target for prostate cancer treatment, a tetrapeptide combinatorial library was screened to first identify an optimal substrate for the protease that was subsequently grafted onto SFTI-1[49]. In the grafting study on the design of antagonists of p53 degradation mentioned above, the epitope was initially discovered in a phage display library.

Once a peptide epitope has been identified, the next step is to select the peptide scaffold, which depends on the functional and/or structural properties that are desired for the final grafted product. Functional properties of scaffolds that have been (or might be) exploited include the tumour targeting ability of cyclic chlorotoxin[16], the protease inhibitory activity of SFTI-1 and MCoTI-II[50], and the cell-penetrating ability of MCoTI cyclotides[16,25,31]. For example, to design potent peptide inhibitors of matriptase, a serine protease that is a therapeutic target for cancer treatment, SFTI-1 and MCoTI-II were chosen as scaffolds to exploit their native ability to inhibit trypsin, a serine protease[50]. As in the example above of a MCoTI-based analogue that successfully antagonized intracellular p53 degradation, other grafting studies have specifically chosen the MCoTI scaffold for its cell-penetrating property[29,38].

The choice of the scaffold can also depend on the structural similarity between the epitope and the scaffold. To design stable inhibitors of the CD2–CD58 protein–protein interaction as potential therapeutics for autoimmune diseases, SFTI-1 and RTD-1 were chosen as scaffolds because their β-sheet structures matched those of the epitopes, and were subsequently used to design grafted peptides with nanomolar activity[33]. With the on-going discovery of natural

scaffolds with novel structures and substantial advances in the *de novo* design of artificial scaffolds[53], the number of scaffolds available for molecular grafting will continue to grow, enabling a greater range of applications.

The next step is to decide where the epitope should be grafted onto the scaffold. The possibilities include: insertion between two existing residues of the scaffold; substitution of one or more residues in a single loop[28,37,40,41]; replacement of residues that span across connected loops[28,49,54]; or replacement of most of the native residues of the scaffold[33,51]. In the latter two cases, epitope residues that overlap with Cys residues of the scaffold might need to be replaced to retain the number of Cys residues of the scaffold and to enable the formation of the intended disulfide connectivity. The choice of grafting site and strategy is typically based on structural similarity of the target site to the active conformation of the epitope. Additionally, the grafting site should allow sufficient exposure of the epitope to the target binding site. Another consideration is the effect of the epitope on the structural and functional integrity of the scaffold. Collectively, these considerations suggest that grafting onto regions of the scaffold that display high flexibility or have high evolutionary variability (e.g. loop 6 of cyclotides) is preferred because these regions might be tolerant to mutation without affecting the fold of the remaining scaffold.

An initial assessment of the quality of the designed peptide can be based on *in silico* predictions of its structure, both alone and in complex with its target. Accurate *in silico* predictions provide an indication of whether steric factors might hinder the interaction between the grafted peptide and its intended target. Computational methods can also be used to rationalize experimental observations. For example, the predicted structural stability of

grafted peptides when bound to the Abl kinase target was shown to correlate well with experimentally determined binding affinities[29].

Once the grafted peptide is designed, it needs to be synthesized using chemical, recombinant, or chemo-enzymatic methods[37,48]. Most commonly, grafted peptides have been made using solid-phase chemical synthesis, which involves first the assembly of the linear reduced precursor, comprising sequences from the scaffold and epitope, followed by oxidization and cyclization to form the final product. Using this approach, synthesis of grafted peptides that are based on topologically simple scaffolds, i.e. SFTI-1, has in our experience been straightforward. However, synthesis of more complex peptides with multiple disulfide bonds, e.g. grafted cyclotides, can be challenging because non-native disulfide bond connectivities may form during oxidation. For example, a peptide comprising just three disulfide bonds has 15 unique connectivities. To address this folding problem, identification of the optimal chemical folding condition to produce the desired grafted peptide is required. We typically start with an alkaline buffer, such as an ammonium bicarbonate buffer at pH ~8.5, as the underlying folding condition, and, if needed, trial the use of additives, such as organic solvents or oxidising agents, to modulate folding yields[55]. An alternative is to use orthogonally protected cysteine pairs to direct disulfide bond formation[15]. The correctly folded product is identified using NMR, initially by assessing the quality of the spectra (well dispersed sharp peaks) followed by a comparison of the chemical shifts of the grafted peptide with that of the parent scaffold. If the product is pure and well-folded, it can then be tested for activity and stability.

In some cases, grafting of an epitope onto a scaffold does not produce the desired outcome – either the grafted peptide cannot be folded correctly or it does not have the desired activity. In these cases, it is often worth revisiting the steps above, i.e. to choose another epitope (that may be an analogue of, or completely different in sequence and structure to the original epitope) or a different scaffold for grafting. For example, grafting of a pro-angiogenic peptide derived from VEGF onto loop 6 of MCoTI-II did not produce a well-folded product even after optimization of oxidation conditions[55], but the same epitope grafted onto a different scaffold, SFTI-1, readily resulted in a peptide that could be efficiently oxidized. Similarly, grafting of alternative pro-angiogenic epitopes (i.e. derived from the laminin α1 chain or osteopontin) onto loop 6 of MCoTI-II resulted in peptides that efficiently oxidized into the desired form. Overall, these results demonstrate that changing the scaffold or epitope can lead to a successful outcome, even if initial attempts fail. Changing the grafting site can also result in success. In an early study, we explored the effect of the epitope sequence and loop choice on folding and activity by grafting a series of overlapping fragments of a MOG-derived epitope onto loops 5, 6, or both, of kalata B1 (ref. 28). Of the 17 analogues synthesized, nine adopted a well-ordered fold. Some fragments were better tolerated by the scaffold when inserted onto loop 5 than loop 6, and vice versa. As an example, grafting of residues 41–47 of MOG onto loop 6 of kalata B1 did not result in a well-folded peptide, whereas grafting of the same epitope onto loop 5 not only led to a product that was well-folded but also had the most potent *in vivo* activity.

Another strategy for improving folding yield or activity is to include linkers that flank the epitope, such as flexible linkers (e.g. containing Gly residues[56]) to relieve over-constraint by the scaffold or structure-stabilizing linkers (e.g. helix-inducing sequences[37,38]) to increase rigidity and exposure. Although increased flexibility and exposure of the grafted epitope may

result in reduced enzymatic stability and sub-optimal activity[57], these properties can also be beneficial for activity[56]. For example, a peptide epitope that is an antagonist of vascular endothelial growth factor A was more active when grafted onto loop 3 than loop 6 of kalata B1, because the loop 3-grafted epitope was less constrained and more able to sample the active conformation[56]. It has also been reported that regions outside the grafted peptide, i.e. belonging to the scaffold, can affect binding affinity to a target protein[57,58]. These additional interactions may provide fortuitous opportunities for optimization.

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An extension of simple molecular grafting is to fuse multiple epitopes onto a scaffold, creating a multivalent analogue that has improved activity, selectivity and/or expanded functionality compared to a variant with only one epitope. So far, grafted peptides that comprise two of the same[19,29] or different epitopes[55] have been designed; these 'dual-graft' studies are highlighted in **Table 1** and **Figure 5**. A dual-graft peptide containing two of the same integrin receptor binding epitopes – one grafted onto each opposing loop of RTD-1 – showed 10-fold higher affinity for the integrins $\alpha v\beta_1$ and $\alpha v\beta_6$ and also higher selectivity than monovalent analogues[19]. Similarly, incorporation of peptidic inhibitors that bind to the substrate site of BCR-ABL kinase into both loops 1 and 6 of MCoTI-II resulted in a dual-graft that was up to 14-fold more potent than the corresponding grafted peptides containing a single insertion[29]. In another recent study, two epitopes targeting separate angiogenesis pathways were grafted onto MCoTI-II, one onto loop 5 and the other onto loop 6 (ref. 55). The dual-graft was more potent at inhibiting blood vessel growth *in vivo*, with a 42% improvement compared to a loop 6-only grafted peptide. These examples are exciting because they demonstrate that molecular grafting has evolved from the original one-epitope-in-one-scaffold strategy, and more adaptations might be possible in the future.

Combinatorial methods for molecular grafting

Molecular grafting is amenable to genetic combinatorial approaches for lead discovery. **Table 2** lists genetic methods that have been adapted to phage-, bacteria- and yeast-based systems. As in chemical design, the choice of scaffold is guided by the application and the desired biopharmaceutical properties. For example, the SFTI-1 scaffold is typically selected for its topological simplicity[59]; MCoTI-II because of its trypsin-inhibitory activity[60]; and kalata B1 because of its high proteolytic stability[61]. For display purposes these scaffolds are typically linearized. For example, to construct cyclotide-based genetic libraries, loops 2 or 6 of cyclotide scaffolds were disconnected to allow for attachment to the display substrate[60-63]. Once an acyclic hit is obtained, it later can be cyclised to improve stability. The choice of grafting site is based on how the grafted peptides might bind to the target, or which regions of the scaffold are most tolerant of substitutions. For example, to identify matriptase inhibitors, a genetic library was constructed by making loop 1 of MCoTI-II the grafting site because that loop binds to the active site of trypsin, an enzyme related to the target[60]. Similarly, thrombin inhibitors were discovered by making loop 6 of kalata B1 the grafting site because this loop exhibits a high degree of sequence variability in the cyclotide family[61]. As illustrated in **Figure 6**, neuropilin-1 and -2 antagonists were discovered by initially making loop 6 of kalata B1 the grafting site; after one round of selection, loop 5 was used because of spatial proximity to loop 6, resulting in nanomolar affinities[62]. This example demonstrates the potential of genetic libraries to discover grafted peptides that contain multiple grafted epitopes that have increased affinity or expanded functionalities compared to single constructs.

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Compared to chemical design of the epitope, there are additional considerations regarding the diversity and coverage of the library. One can start with a fully randomized library. For example, to select for thrombin inhibitors, a seven-amino acid peptide library where each position of the epitope was allowed to vary was created within loop 6 of kalata B1 and transformed into *E. coli*[61]. The library comprised 6×10^9 transformants, representing ~18% coverage of the theoretical library diversity (3.4×10^{10} sequences). Alternatively, a grafted peptide can be used for optimization by combinatorial display. To identify integrin $\alpha v\beta_6$ binders, epitopes found from phage display were first grafted onto MCoTI-II to identify optimal starting grafted peptides, which contained either 13 or 14 residues in loop 1 (ref. 63). Lead peptides were then used to design a focused library by fixing five of the residues in loop 1 and allowing the others to vary, creating a combined library of $\sim 1 \times 10^7$ clones. After a second library was created and screened, grafted peptides with nanomolar affinities and receptor selectivity were found. We note that it is important to synthesize and structurally characterize identified hits from library screens, because displayed peptides might adopt non-scaffold-like conformations[61].

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Future perspectives

Grafting onto disulfide-rich scaffolds has been very successful in achieving proof-of-concept status in more than two dozen pharmaceutically relevant studies to date, many showing enhanced stability of the grafted peptide as well as improved activity (in the nanomolar range) and/or selectivity, with confirmation of efficacy in animal disease models. Two studies have demonstrated modulation of intracellular activity[37,38] and four have demonstrated orally delivered activity of the grafted peptide[15,32,40,41]. Thus, molecular grafting has helped overcome some of the challenges faced in translation of peptides to the clinic. However, others still require attention, including: (*i*) the need to improve the biopharmaceutical

properties of grafted peptides; (*ii*) the need for information on the potential immunogenicity of grafted peptides; and (*iii*) the development of large-scale production approaches for disulfide-rich cyclic peptides. These challenges might account for why no grafted peptide has yet progressed to human clinical trials, but the potential for this to occur in the next few years is certainly there.

It is encouraging that four chemically diverse grafted peptides have so far exhibited oral activity[15,32,40,41], suggesting that molecular grafting might partly address the oral administration challenge. However, we know very little about why these peptides are orally active ~~as well as~~or about their pharmacodynamics and pharmacokinetic properties. For now, it might be worthwhile to focus on therapeutic applications that side-step the delivery hurdle and thus allow for fast-tracking of translation[64]. For example, grafted peptides that have been developed into imaging agents with *in vivo* selectivity[63] could be administered intravenously.

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Compared to linear cell-penetrating peptides, cyclic disulfide-rich scaffolds are potentially more desirable because of their enhanced stability. So far, two studies[37,38] have demonstrated that cyclic grafted peptides can modulate intracellular pathways, and others have shown that cellular uptake can be enhanced[27,39]. Despite these advances, there are still many uncertainties regarding ~~XXXX~~transport and intracellular distribution, particularly relating to endosomal release.

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Will grafted scaffolds be immunogenic? We believe that their small size (<50 amino acids) and protease resistance (and hence resistance to immunological processing and display) makes them low risk in this regard. Nevertheless, the fact that some scaffolds can be modified to modulate the immune system[28,33,41] does suggest the possibility for an

immunogenic response, but it also suggests that other modifications might engender immunogenic silence.

What about production costs? Currently, production of milligram to gram quantities of grafted peptides is relatively straightforward using solid phase peptide synthesis, but large-scale (kg to ton) production using this approach is likely not to be cost-effective. The production of cyclic disulfide-rich peptides is complicated by the need to obtain the correct disulfide connectivity as well as cyclization of the backbone. Hybrid methods that incorporate biosynthetic reactions derived from nature[14,65,66] might thus be of help. For example, use of efficient cyclization enzymes, such as the recently discovered butelase[67] or OaAEP1 (ref. 68), has the potential to facilitate large-scale production of grafted cyclic peptides. Furthermore, since many of the scaffolds we have described here are natural plant-based peptides, it makes sense that in the future crop plants might be used as biofactories for cheaply producing peptide-based pharmaceuticals. Our laboratory is already making progress in this field, which offers not only the possibility of harvesting and refining pharmaceutical products from crop plants, but in some cases also using the plants as edible medicines. One can imagine seeds containing pharmaceutical peptides being 'biopills' that might be an alternative in some cases to tablets, or medicinal teas that might be made from leaf-expressed grafted macrocycles.

What about non-pharmaceutical applications? Our survey of the grafting studies completed so far highlights aspects of molecular grafting that are under-exploited and thus could be explored further. Clearly, most studies have focused on therapeutic applications, particularly on the development of stabilized inhibitors. Few studies have explored the potential of cyclic grafted peptides as diagnostics, in nanotechnology or as agricultural agents, despite the

potential that peptides in general have demonstrated use in those applications[69-72]. With increasing awareness of environmental impact of traditional chemical pesticides, stable peptidic pesticides offer a promising alternative that is definitely ripe for the picking.

With these broader challenges of translation in mind, we circle back to the focus of this Perspective – the molecular grafting process. Certainly, improvements to the process will accelerate discovery of grafted peptide drugs. Currently, we know that not all grafted peptides fold into the desired conformation, and so more detailed understanding of how grafting affects folding of disulfide-rich peptides would be beneficial. Perhaps the use of accurate *de novo* modelling methods for peptides[53] might be useful here to assess folding. Additionally, recent advances that allow for facile incorporation of sophisticated chemical modifications to libraries will expand their chemical diversity, and therefore increase the potential of combinatorial approaches[73,74]. We also note that several recent studies have demonstrated the benefits of multiple grafts[19,29,55], an aspect of grafting that could be further developed.

We conclude by noting that pharmaceutical companies appear to be amenable to a call-to-action in the peptide field. All of the large pharma companies have re-invigorated their peptide chemistry interests, with many doing this in collaboration with academic labs and smaller biotechnology companies. With an increasing corpus of researchers in the field one can expect a bright future for peptide-based drug design, and we believe that macrocycles will be front and center of this effort.

Acknowledgements

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Competing financial interests

The authors declare no competing financial interests.

Figure Legends

Figure 1: Selected cyclic disulfide-rich peptide scaffolds and their sequence diversity. (**a**) The range of scaffolds discussed in this Perspective. Representative structures of each scaffold class are shown~~. The~~ with cysteine residues ~~are~~ labeled with Roman numerals. (**b**) Sequences of cyclic disulfide-rich peptide scaffolds: sunflower trypsin inhibitor-1 (SFTI-1), rhesus θ-defensin-1 (RTD-1), cyclic Vc1.1 (cVc1.1), kalata B1 (kB1), and cyclic chlorotoxin (cChltx). Also shown is the disulfide bond connectivity, loop nomenclature and sequence diversity of each loop, the latter represented by a circled number that shows the number of alternative residue substitutions found at the indicated site. Sequence diversity was determined from sequences deposited in CyBase (cybase.org.au) or UniProt (uniprot.org).

Figure 2: Molecular grafting of epitopes onto scaffolds. In molecular grafting, a peptide epitope (which has desired biological activities) is 'grafted' onto a constrained peptide (which has desired biopharmaceutical properties), resulting in a new grafted peptide that has the desired properties of both chemical inputs. The epitope can be chosen from a range of sources, such as (*i*) naturally-occurring peptides or fragments of an interacting protein, (*ii*) chemical peptide libraries or (*iii*) recombinant peptide libraries. The scaffold can be chosen from a panel of cyclic disulfide-rich peptides, such as those shown in **Figure 1**.

Figure 3: Applications of molecular grafting. (**a**) Molecular grafting onto a cyclic peptide scaffold can be used to stabilize a biologically-active peptide sequence. In a recent study, the kalata B1 scaffold was used to stabilize peptide epitopes from myelin oligodendrocyte glycoprotein for the treatment of multiple sclerosis[28]. (**b**) Molecular grafting can also be used to deliver peptides into cells. ~~Camarero and co-workers grafted a~~A peptide sequence from

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p53 grafted onto MCoTI-I~~, and showed that the grafted peptide~~ could inhibit a protein–protein interaction interface of intracellular Hdm2–HdmX[37]. (**c**) Grafted peptides could potentially be delivered via oral administration. ~~Tam and co-workers demonstrated that k~~Kalata B1 grafted with a bradykinin receptor antagonist had orally delivered activity in an animal model of disease[40].

Figure 4: The molecular grafting process in rational drug design. The flowchart shows the typical decision-making process that is employed to discover a novel grafted peptide drug lead. The process involves an initial design phase, in which an appropriate peptide epitope and peptide scaffold are chosen, followed by the grafting site. A designed peptide is then synthesized and purified, and its structure assessed by NMR to determine whether it is well folded. If the purified product is also stable and active, then the grafted peptide is a potential lead compound for further testing or optimization.

Figure 5: Design of multivalent peptides by molecular grafting. The concept of molecular grafting has evolved since original studies demonstrating proof-of-concept, in which one epitope was grafted onto a signal loop of the scaffold to engineer first-generation grafted peptides[75]. Since then, more than one epitope has been grafted onto a scaffold; for example two epitopes with the same activity[29] has been grafted onto MCoTI-II to increase valency (second generation), and two epitopes with different activities[55] have been grafted to target multiple pathways (third generation). The grafted peptides comprising two epitopes are referred to as 'dual-grafts' and these are highlighted in **Table 1**. It is theoretically possible to design grafted peptides with multiple functions by grafting an epitope that targets the disease cell as well as another epitope that modulates the activity of the protein target, and these might represent the fourth generation of grafted peptides.

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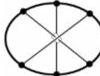
Figure 6: A combinatorial library approach to molecular grafting. Grafted peptides can be discovered by using a selected scaffold to design a library of variants that are then genetically encoded and screened for activity. Here, neuropilin-1 and -2 antagonists were discovered by making bacterial display libraries based on the kalata B1 scaffold (top; sequence also shown)[62]. To allow for attachment of each variant to a bacterial membrane protein (purple hexagon), loop 2 of kalata B1 was disconnected. Initially, the entire loop 6 was made variable (white circles with X) and then after one round of sorting, loop 5 and selected residues in other loops were randomized. Active hits were synthesized by solid-phase peptide synthesis and used to confirm nanomolar potency of grafted peptides.

Tables

Table 1. Grafting of cyclic disulfide-rich peptide scaffolds by chemical design.

Scaffold[a]	Epitope Size[b]	Activity[c]	Target class	Application
SFTI-1				
	3[d]	Kallikrein-related peptidase inhibitor[76]	Enzyme	Cancer
	3	NF-κB inhibitor[64]	Protein-protein interaction	Inflammatory bowel disease
	9	Angiogenic[30]	Cell-surface receptor	Cardiovascular and wound healing
	6	Matriptase inhibitor[50]	Enzyme	Cancer
	3[d]	Matriptase inhibitor[77]	Enzyme	Cancer
	7	Anti-angiogenesis[78]	Cell-surface receptor	Inhibiting tumour progression
	15[d]	Post-synaptic density-95 binding[48]	Protein-protein interaction	Neurological diseases
	5[d]	Kallikrein-related peptidase inhibitor[79]	Enzyme	Cancer
	9[d]	Tau aggregation inhibitor[54]	Protein-protein interaction	Alzheimer's disease
	10[d]	CD2:CD58 inhibitor[e, 33]	Cell-surface receptor	Autoimmune diseases
	9	Bradykinin receptor antagonist[32]	Cell-surface receptor *(oral activity)*	Chronic pain and inflammatory pain
cyclic conotoxin				
	6	Cyclization[15]	Cell-surface receptor *(oral activity)***	Neuropathic pain
	16[d]	GLP-1 receptor agonists[f, 51]	Cell-surface receptor	Diabetes
θ-defensin				
	6[d]	Integrin receptor binding[19]	Cell-surface receptor *(dual-graft)*	Anti-tumour and anti-cancer
	12[d]	CD2:CD58 inhibitor[33]	Cell-surface receptor	Autoimmune diseases
	7[d]	Tumour targeting and survivin binding[80]	Protein-protein interaction *(dual-graft)*	Anti-tumour and anti-cancer
kalata B1				
	3[d]	Proof-of-concept[g, 81]	N/A	Wide range of diseases
	9	VEGF-A antagonist[h, 56]	Protein-protein interaction	Inhibiting tumour progression
	6	Melanocortin receptor 4 agonist[34]	Cell-surface receptor	Obesity
	9	Bradykinin B_1 receptor antagonist[40]	Cell-surface receptor *(oral activity)* **	Chronic pain and inflammatory pain
	13[d]	Immuno-modulation[28]	Protein-protein interaction	Multiple sclerosis
	1	Lymphocyte proliferation inhibitor[41]	Protein-protein interaction *(oral activity)*	Multiple sclerosis

MCoTI				
	3	FMDV 3C protease inhibitor[i, 75]	Enzyme	Foot-and-mouth disorders
	3	β-tryptase and elastase inhibitor[82]	Enzyme	Inflammation disorders
	9	VEGF receptor agonist[h, 30]	Cell-surface receptor	Angiogenesis
	16	Cytokine receptor CXCR4 antagonist[j, 57]	Cell-surface receptor	Anti-HIV
	7	Matriptase inhibitor[50]	Enzyme	Anti-tumour
	18	Hdm2/X antagonist[k, 37]	Protein-protein interaction *(intracellular)* **	Anti-tumour
	19[d]	BCR-ABL tyrosine kinase inhibitor[l, 29]	Enzyme *(dual graft)*	Chronic myeloid leukemia
	9[d]	Cell penetration[39]	Cell-surface interactions	Wide range of intracellular targets
	6	MAS1 receptor[m, 31]	Cell-surface receptor	Cancer and myocardial infarction
	21	SET Antagonist[38]	Protein-protein interaction *(intracellular)*	Cancer
	11[d]	Somatostatin and PEDF receptor[n, 55]	Cell-surface receptors *(dual graft)***	Cancer
	7	Tumour targeting[80]	Protein-protein interaction *(dual graft)*	Anti-tumour
	5[d]	Factor XIIa inhibitor[83]	Enzyme	Cardiovascular disease
chlorotoxin				
	10[d]	Cyclization[16]	Cell-surface receptor	Tumour-imaging

[a] Examples of cyclic disulfide-rich peptide scaffolds include those with one disulfide bond (e.g. sunflower trypsin inhibitor-1), two disulfide bonds (e.g. cyclic Vc1.1), three disulfide bonds in a laddered arrangement (e.g. rhesus θ-defensin-1), three disulfide bonds in a knotted arrangement (e.g. kalata B1, *Momordica cochinchinensis* trypsin inhibitors-I and -II), and four disulfide bonds (e.g. chlorotoxin). [b] Largest epitope used for grafting. [c] Reference citations are provided. [d] non-contiguous sequence. [e] CD2/CD58: cell-cell adhesion proteins. [f] GLP-1: glucagon-like protein-1. [g] Proof-of-concept to show loops can be modified or grafted. [h] VEGF: vascular endothelial growth factor. [i] FMDV: foot and mouth disease virus. [j] CXCR4: C-X-C chemokine receptor type 4. [k] Hdm2: Human double minute 2. [l] BCR-ABL: breakpoint cluster-Abelson. [m] MAS1 mas-related G protein-coupled receptor A. [n] PEDF: pigment epithelium-derive factor. ** highlights key examples of grafting studies demonstrating increased oral activity and intracellular delivery of designed peptides, as well as exploring multivalent display of epitopes.

Table 2. Grafting of cyclic disulfide-rich peptide scaffolds by library-based strategies.

Scaffold[a]	Epitope Size[b]	Activity[c]	Target class	Application	Library Strategy[d]
SFTI-1					
~~(1 SS)~~	8	Delta-like ligand 4 binding[59]	Membrane protein	Tumour targeting	P[f,g]
	6[e]	Kallikrein-related peptidase inhibitor[49]	Enzyme	Cancer	S
	5[e]	Cathepsin G[84]	Enzyme	Chronic inflammatory disorders	S
kalata B1					
~~(3 SS)~~	7	Thrombin inhibitor[61]	Enzyme	Cardiovascular disease	B[f]
	14[e]	Neuropilin-1 and -2 antagonist[62]	Cell-surface receptor	Angiogenesis	B[f]
MCoTI					
~~(3 SS)~~	22[e]	Integrin receptor binding[63]	Cell-surface receptor	Pancreatic cancer detection	Y[f]
	17[e]	Matriptase inhibitor[60]	Enzyme	Anti-tumour	Y[f]
	17[e]	CTLA-4 binding[h, 85]	Cell-surface receptor	Metastatic melanoma	Y[f]
	8	α-synuclein aggregation inhibitor[86]	Protein-protein interaction	Parkinson's disease	Y

[a] Examples of cyclic disulfide-rich peptide scaffolds include those with one disulfide bond (e.g. sunflower trypsin inhibitor-1) and three disulfide bonds in a knotted arrangement (e.g. kalata B1, *Momordica cochinchinensis* trypsin inhibitors-I and -II). [b] Largest epitope used for grafting. [c] Reference citations are provided. [d] Strategy type: Phage display (P), Synthetic library (S), Bacterial display (B), and Yeast display (Y). [e] non-contiguous sequence. [f] Some display technologies require linearized analogues. [g] Phage library was used but no hits discovered. [h] CTLA-4: cytotoxic T lymphocyte-associated antigen 4.

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